Supporting Information

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SI Materials and Methods

Plasmid and Strain Construction. All plasmids and strains are listed in Table S1. The concentrations of antibiotics used in the selections were as follows: kanamycin (Kan) 25 μg/mL, ampicillin (Amp) 125 μg/mL, chloramphenicol (Cam) 20 μg/mL; tetracycline (Tet) 10 μg/mL. Strains were grown in Luria-Bertani (LB) medium (1).

The majority of deletion mutants in this study originated from the Keio collection, an *E. coli* library of nonessential insertion-deletion mutants where each target gene is replaced with a Kan-resistance (Kan^R) cassette flanked by FLP-recognition target sites (2). P1 lysates prepared from the Keio mutants of interest were transduced into our wild-type MC4100 strain, JCM158 (Table S1) according to standard methods (1). The resulting strains were transformed according to protocol with pCP20, which provides FLP recombinase required for Kan^R removal (3). The resulting deletion mutants were subsequently cured of pCP20 at 42 °C.

The Keio library does not include an *mlaB*::*kan* (*yrbB*::*kan*) deletion, therefore *mlaB* is presumed to be an essential gene (2). However, we noticed that the upstream *mlaC*::*kan* mutation resulted in a slow growth phenotype that was abolished upon removal of the Kan^R cassette. The transcription of the Kan^R gene is oriented in the same direction as the *mla* operon, and we suspected that the *mlaC*::*kan* mutation could result in increased transcription of a downstream gene, either *mlaB* or *yrbA*, which is toxic when overexpressed. The *yrbA* gene is paralogous to *bolA*, which has been shown to result in a rounding of the cell upon overexpression (4). We constructed a *mlaB* deletion mutation using recombineering as previously described (5), in which the *mlaB* ORF was replaced with a chloramphenicol resistance (Cam^R) cassette transcribed in the opposite orientation relative to *yrbA*. The Cam^R cassette was amplified from the pKD3 plasmid with regions flanking the *mlaB* locus (Table S2). The resulting PCR product was electroporated into strain DY378 (5) and plated onto LB + Cam at 30 °C. The *mlaB*::*cam* allele was subsequently introduced into the wild-type strain by P1 transduction. No growth defect was detected, and the Cam^R cassette was subsequently removed using the pCP20 plasmid as described above.

The *pldA* and *pagP* genes were amplified from MC4100 genomic DNA, using primers detailed in Table S2. The products were digested with DraI (note that the *pagP* PCR product contained a suitable DraI site near its C terminus) and ligated into pBR322 that had been digested with ScaI and treated with calf-intestinal alkaline phosphatase (New England Biolabs). The resulting constructs are not Amp^R as the ScaI site interrupts the *bla* gene, therefore transformants were selected on LB + Tet. DNA sequencing confirmed that both genes were free of mutation and inserted in the same orientation relative to the upstream plasmid-borne *bla* promoter to ensure expression. Finally, qRT-PCR tests were performed to confirm adequate gene expression (see below).

Verification of MlaD Localization. The SignalP version 3.0 neural network analysis server utilizes algorithms that distinguish predicted transmembrane signal sequences from processed secretory proteins (6). The indicators for MlaD signal cleavage (C-max and Y-max prediction scores) are well below the threshold currently used to discriminate processed signals in Gram-negative bacteria (the MlaD C-max and Y-max scores = 0.097 and 0.289, respectively; SignalP 3.0 cutoff values = 0.52 and 0.33, respectively), suggesting that the MlaD signal sequence has a low probability of signal sequence processing. We note that many public databases currently predict that MlaD is a periplasmic protein, but these predictions appear to be based on an obsolete version of the SignalP algorithm.

To verify the computational prediction, we amplified the *mlaD* gene from our wild-type strain (primer sequences are given in Table S2) and cloned the full-length *mlaD* gene into pET28a (Novagen) using NcoI and HindIII restiction enzymes (New England Biolabs), resulting in a C-terminal His-tag fusion construct. The (pET28a::*mlaD*-His) plasmid was transformed into BL21 (pLysE) cells (Promega). Cells were grown in 20 mL of LB + Cam/Kan at 37 °C to an optical density (OD) of 0.5 at 600 nm. Cultures were cooled to room temperature and plasmid expression was induced with 0.1 mM IPTG. Cultures continued to incubate overnight at room temperature with shaking and subsequently pelleted and resuspended in 4 mL of 10 mM Tris (pH 7.5), 5 mM EDTA (pH 7.5), 1 mM PMSF, 0.05 mg/mL DNase, and 0.1 mg/mL lysozyme. This suspension was lysed via 3 passages through a French press apparatus (14,000 p.s.i.). Unlysed cells were removed through centrifugation at 3,000 × *g* at 4 °C. The lysed sample was subsequently fractionated using an isopycnic sucrose gradient as previously described (7, 8). The gradient was disassembled from the top in 3-mL fractions, and the density of each fraction was estimated and used to identify the fractions enriched in soluble, IM and OM proteins (Fig. S1).

Fractions were diluted 1:1 in 2× SDS sample buffer and boiled for 10 min. Samples (15 μL) were loaded onto a 12% acrylamide gel (9) and electrophoresed 1.5 h at 120 V. Gels were transferred onto nitrocellulose membranes using a semidry apparatus (Bio-Rad) for 1.5 h at 10 V. Membranes were blocked overnight in blocking buffer and incubated with either Penta-His HRP Conjugate Kit at 1:2,000 as recommended by the manufacturer (Qiagen) or with αLptD (αlmp) antibody as described (10) to serve as a marker for soluble IM and OM proteins. Bands were visualized using ECL chemiluminescent reagent (GE Healthcare Life Sciences) and HyBlot CL film (Denville Scientific).

Identification of the Site of miniTn10 Transposon Insertion. The location of the miniTn10 insertion linked to the Δ*mlaC* SDS-$EDTA^R$ suppressor mutation was identified using a 2-step PCR procedure (12). Round 1 used the ARB1 degenerate primer containing a 5′ sequence tag and a second primer internal to the miniTn10 transposon (TetA-out), in a thermal reaction cycle as follows: 95 °C 5 min; 5 cycles of 94 °C for 30 sec, 30 °C for 30 sec, 72 °C for 1.5 min; 30 cycles of 94 °C for 30 sec, 45 °C for 30 sec, 72 °C for 2 min; and extension at 72 °C for 5 min. For the 2nd round of PCR, we used a primer that recognized the 5′ tag used in round 1, ARB2, with a second primer that recognized the rightmost end of the miniTn10 transposon (TetA-seq). One one-hundredth of the reaction from round 1 was used as a template in round 2 using a cycling protocol as follows: 29 cycles [94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1.5 min]; and 72 °C for 5 min. PCR products were run on a 1% agarose gel and the resulting band was excised and purified using a Qiagen PCR purification kit. Sequencing was performed using Genewiz sequencing services and the TetA-seq primer. Primer sequences are given in Table S2.

Quantitative Real-Time PCR (qRT-PCR). Cultures were grown overnight in LB and diluted 1:100 in fresh medium. Once cultures reached an $OD_{600} \approx 0.5$, 1 mL was spun for 1 min at 10,000 ×

g and total RNA was immediately extracted from the cell pellets using the RiboPure™ kit according to the manufacturer's instructions (Applied Biosystems/Ambion). RNA purity and concentration were determined using a NanoDrop 1000 (Thermo Scientific). cDNA was subsequently amplified from a 1:10 dilution of RNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). The remaining RNA in each 20 μL reaction was hydrolyzed by the addition of 15 μL of 1 N NaOH and incubated at 65 °C for 30 min. Reactions were neutralized with 15 μL of 1 N HCl and cleaned using MinElute PCR purification spin columns according to protocol (Qiagen). cDNA purity was verified using the NanoDrop 1000 before proceeding with the qRT-PCR procedure. Reactions were set up in a MicroAmp™ Fast Optical 96-well reaction plate, and Syber Green PCR Master Mix was added to each reaction as suggested by the manufacturer (Applied Biosystems). qRT-PCR reactions were performed using an ABI Prism 7900 real-time PCR system with ABI Prism SDS software (v2.1) (Applied Biosystems). Experiments were performed at least twice and data points from individual experiments were averaged from triplicate reactions. The target transcripts were quantified using *ompA* transcript as an internal control.

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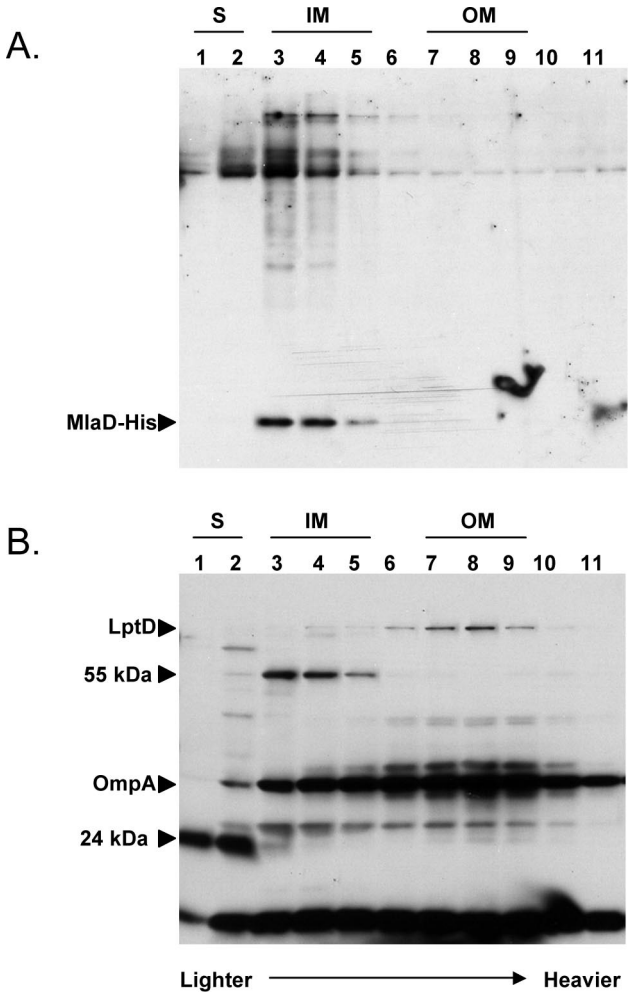

Fig. S1. Western blot of sucrose-gradient fractions. Fractions increase in density from left to right and are blotted with (*A*) anti-His antibody or (*B*) anti-LptD antibody. MlaD-His in *A* is enriched in lanes 3, 4, and 5, which correspond to the same fractions as the 55-kDa IM protein of unknown identity (10) in *B*. Other useful markers in panel *B* include the 24-kDa band enriched in the soluble fractions (S, lanes 1 and 2), and the OM proteins OmpA and LptD, which are mostly enriched in lanes 7, 8, and 9.

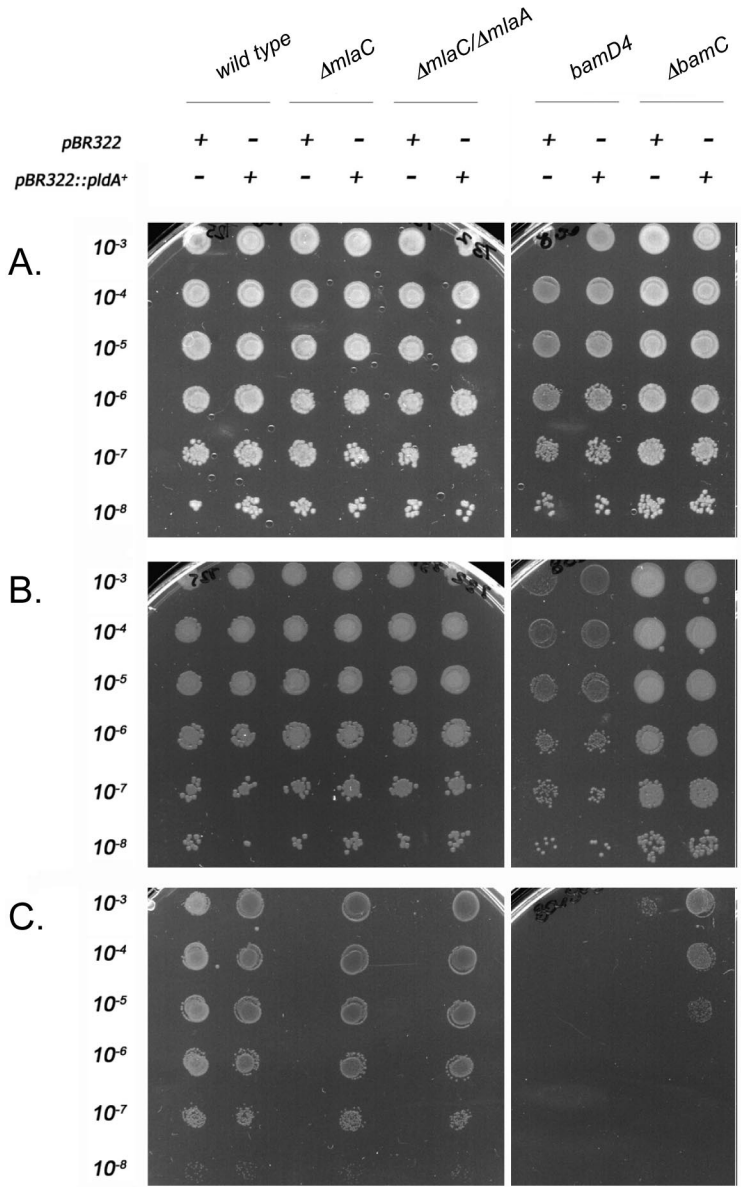

Fig. S2. OM permeability phenotypes of various strains expressing multicopy *pldA.* Colony forming units are shown and approximate dilutions are given on the *Left* side of the panels. (*A*) LB; (*B*) LB + SDS-EDTA (0.5%–0.55 mM); (*C*) LB; + SDS-EDTA (0.5%–1.1 mM). Plus and minus signs refer to the presence or absence of either pBR322 or pBR322::*pldA.*

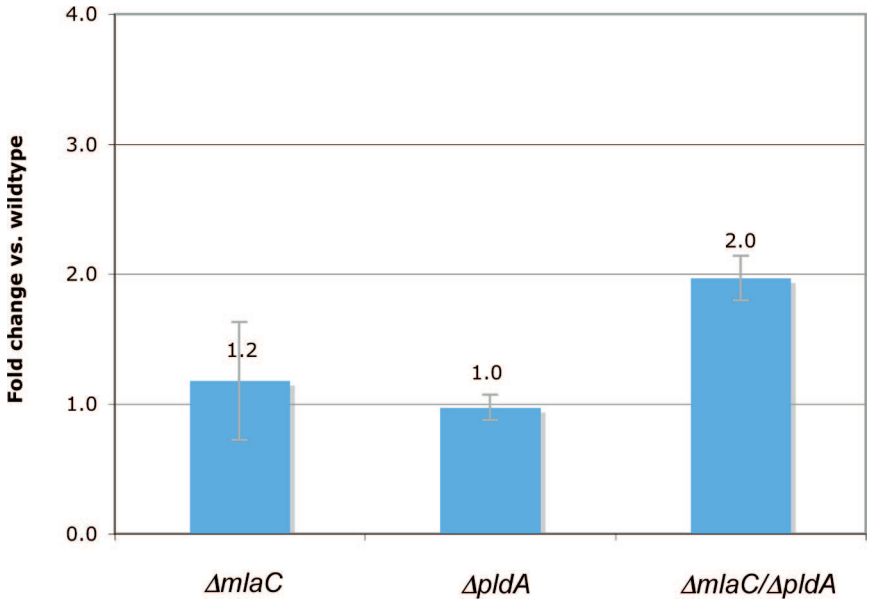

Fig. S3. Quantification of *pagP* transcript in various strains by qRT-PCR. Cultures were grown in LB to an equivalent density in late-exponential phase. *pagP* transcript levels were normalized to an internal control of *ompA* transcript. Final *pagP* values are given as relative change compared with the wild-type strain. Data points are the average of triplicate samples from 2 independent experiments.

Table S1. Plasmids, strains, and phage used in this study

Bacterial strains	Genotype and relevant features	Reference
MC4100	F$^-$ *araD139* Δ(*argF-lac)U169 rpsL150 relA1 flb5301 deoC1 ptsF25 thi*	(1)
DY378	W3110 λcI*857* Δ(*cro-bioA*)	(13)
BL21 (pLysE)	IPTG inducible expression system	Promega
JCM158	MC4100 *ara$^{r/-}$; spontaneous arabinose resistant*	(14)
JCM304	JCM158 *bamC*::*kan*	(15)
JCM344	JCM158 *bamD4*::*cam*	(15)
JCM528	JCM158 Δ*mlaC*	This study
JCM529	JCM158 Δ*mlaD*	This study
JCM530	JCM158 Δ*mlaE*	This study
JCM531	JCM158 Δ*mlaF*	This study
JCM718	JCM158 Δ*mlaA*	This study
JCM720	JCM158 Δ*mlaA* Δ*mlaC*	This study
JCM735	JCM158 Δ*pldA*	This study
JCM736	JCM158 Δ*pldA* Δ*mlaC*	This study
JCM741	JCM158 Δ*pagP*	This study
JCM742	JCM158 Δ*pagP* Δ*mlaC*	This study
JCM743	JCM158 Δ*pagP* Δ*pldA*	This study
JCM744	JCM158 Δ*pagP* Δ*mlaC* Δ*pldA*	This study
JCM745	JCM158 Δ*mlaA*	This study
JCM751	JCM528 suppressor class 1; A → G −81 *pldA*	This study
JCM752	JCM528 suppressor class 2; IS5 −68 *pldA*	This study
JCM753	JCM528 suppressor class 3; IS2 −61 *pldA*	This study
JCM754	JCM528 suppressor class 4; IS1 −69 *pldA*	This study
JCM775	JCM158 Δ*mlaB*	This study
Plasmids		
pBR322	Cloning vector; Tet^R, Amp^R	(16)
pCP20	*FLP$^+$*, λ cI857+, λ p_R Rep^{ts}, Amp^R, Cam^R	(3)
pKD3	Contains Cam^R cassette	(5)
pET28a	Cloning vector, Kan^R	Novagen
pET28a::*mlaD*	IPTG-inducible *mlaD-His*	This study
pBR322-*pldA*	Tet^R	This study
pBR322-*pagP*	Tet^R	This study
Phage		
λNK1323	mini-Tn10 transposon	(11)
P1 vir		(1)

Table S2. Primers

Primer name	Sequence*
Recombineering primers	
mlaB-P1 Fwd	5′- CTGAAATCGATTTCTCAACAGAAAATCACTCTGGA AGAGAAAAAATAATGGTGTAGGCTGGAGCTGCTTC −3′
mlaB-P2 Rev	5′- TAACGCCATATCCGGCCTGAAAAAATTTAACGAGG CAGAACATCAGCAGGCATATGAATATCCTCCTTAG −3′
Cloning primers	
5′ mlaD-His-NcoI	5′- ATAccATGgAAACGAAAAAAAATG −3′
3′ mlaD-His-HinDIII	5′- ATAaagcttTTTCGTTGTACCCAC −3′
pldA(−200) Fwd-DraI	5′- TTTTTAAAGGCCAGCTGTGCGAAC −3′
pldA(+1223)Rev-DraI	5′- TTTTTAAAGCGGTGAAACAACCACGG-3′
pagP(−50)Fwd-DraI	5′- TTTTTAAAAGCTTTGCTATGCTAGT −3′
pagP(+806)Rev	5′- GTTACCTTTAATCTTAGACATC −3′
Inverse PCR primers	
ARB1	5′- GGCCACGCGTCGACTAGTAC$(N)_{10}$GATAT −3′
TetA-out	5′- CCTTCATGTTAACCCCTCAAGCTCAGGGG −3′
ARB2	5′- GGCCACGCGTCGACTAGTAC −3′
TetA-seq	5′- ACCTTTGGTCACCAACGCTTTTCC −3′
qRT-PCR primers	
pagP(+299)Fwd	5′- AGGACTCGTGGAACAAATGG −3′
pagP(+400)Rev	5′- CGGTGAATCCCAGACCTAAA −3′
pldA(+647)Fwd	5′- AGCTTAAAATCGGCTATCACCTC −3′
pldA(+751)Rev	5′- TCGGGTAACTTAAGCCTAACTCC −3′
5′ ompA RT	5′- TACGCGATCACTCCTGAAATC −3′
3′ ompA RT	5′- GTAGGAAACACCCAGGCTCA −3′

*Relevant restriction sites are underlined.